

This Page Is Inserted by IFW Operations
and is not a part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

IMAGES ARE BEST AVAILABLE COPY.

**As rescanning documents *will not* correct images,
please do not report the images to the
Image Problem Mailbox.**

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



35

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

| | | | |
|---|--|---|--|
| (51) International Patent Classification 5 : A61K 9/16 | | A1 | (11) International Publication Number: WO 93/18754 (43) International Publication Date: 30 September 1993 (30.09.93) |
| (21) International Application Number: PCT/US93/02245 (22) International Filing Date: 11 March 1993 (11.03.93) | | (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). | |
| (30) Priority data: 849,186 11 March 1992 (11.03.92) US | | Published <i>With international search report.</i> | |
| (71) Applicant: PHARMACEUTICAL DISCOVERY CORPORATION [US/US]; 7 Westchester Plaza, Elmsford, NY 10523 (US). | | | |
| (72) Inventors: FELDSTEIN, Robert ; 1396 Park Lane, Pelham, NY 10803 (US). GLASS, John ; 94 Highland Dawn, Shoreham, NY 11786 (US). STEINER, Solomon, S. ; 24 Old Wagon Road, Mount Kisco, NY 10549 (US). | | | |
| (74) Agents: PABST, Parea, L. et al.; Kilpatrick & Cody, 1100 Peachtree Street, Suite 2800, Atlanta, GA 30309-4530 (US). | | | |

(54) Title: SELF-ASSEMBLING DIKETOPIPERAZINE DRUG DELIVERY SYSTEM

(57) Abstract

Drug delivery systems have been developed based on the formation of diketopiperazine (or analogs) microparticles. In the preferred embodiment the microparticle is stable at low pH and disintegrates at physiological pH, and is particularly useful for oral drug delivery. In the most preferred embodiment the microparticles are formed in the presence of the drug to be delivered, for example, insulin or heparin. The diketopiperazine synthetic intermediates are preferably formed by cyclodimerization to form diketopiperazine derivatives at elevated conditions under dehydrating conditions, then precipitated with drug to be incorporated into microparticles.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | |
|----|--------------------------|----|--|----|--------------------------|
| AT | Austria | PR | France | MR | Mauritania |
| AU | Australia | CA | Gabon | MW | Malawi |
| BB | Barbados | GB | United Kingdom | NL | Netherlands |
| BE | Belgium | GN | Guinea | NO | Norway |
| BF | Burkina Faso | GR | Greece | NZ | New Zealand |
| BC | Bulgaria | HU | Hungary | PL | Poland |
| BJ | Beira | IE | Ireland | PT | Portugal |
| BR | Brazil | IT | Italy | RO | Romania |
| CA | Cacaua | JP | Japan | RU | Russian Federation |
| CF | Central African Republic | KP | Democratic People's Republic of Korea | SD | Sudan |
| CC | Congo | KR | Republic of Korea | SE | Sweden |
| CH | Switzerland | KZ | Kazakhstan | SK | Slovak Republic |
| CI | Costa d'Ivoire | LJ | Liechtenstein | SN | Senegal |
| CM | Cameroon | LK | Sri Lanka | SU | Soviet Union |
| CS | Czechoslovakia | LU | Luxembourg | TD | Chad |
| CZ | Czech Republic | MC | Mongaco | TC | Togo |
| DE | Germany | MG | Madagascar | UA | Ukraine |
| DK | Denmark | ML | Mali | US | United States of America |
| ES | Spain | MN | Mongolia | VN | Viet Nam |
| FI | Finland | | | | |

SELF-ASSEMBLING DIKETOPIPERAZINE DRUG DELIVERY SYSTEM**Background of the Invention**

This invention is generally in the area of drug delivery systems and is particularly related to reversible microencapsulation of drugs by certain 2,5-diketo-derivatives of piperazine.

Delivery of drugs has been a major problem for many years. It is particularly a problem when the compound to be delivered is unstable under the conditions encountered when administered to the patient, prior to reaching its targeted location. For example, it is preferable in many cases to administer drugs orally, especially in terms of ease of administration, patient compliance, and decreased cost. However, many compounds are ineffective or exhibit low or variable potency when administered orally. Presumably this is because the drugs are unstable to conditions in the digestive tract or because they are inefficiently absorbed.

The field of oral drug delivery covers a broad range of delivery systems ranging from simple mechanical carriers such as pressed tablets which transport compounds that can be safely and efficiently delivered through the stomach, to enteric coatings which delay the release of the encapsulated compound to later in the digestive process and lower in the gastrointestinal tract.

A variety of enteric coatings have been used to encapsulate and protect the drug prior to reaching the small intestine. In some cases these are effective. However, there are drugs that are also unstable to the conditions present in the small intestine and therefore must be administered in much higher dosages if the drug is to be released in the small intestine for an effective amount to penetrate to the bloodstream. In these cases, it is necessary to have a mechanism whereby the coating is not only stable to the conditions present in the digestive tract, as well

-2-

as to the conditions under which it is stored prior to administration to the patient, but which allows the encapsulated drug to pass into the bloodstream.

Other factors in drug delivery system design include the requirements that the system must be non-toxic, non-reactive with the drug to be delivered, not too expensive or difficult to manufacture, formed of readily available components, and consistent with respect to final composition and physical characteristics, including stability and release rate. The system must also be formed of materials that are easily removed by normal metabolic processes.

A number of different systems have been proposed, most based on peptides or biodegradable natural or synthetic polymers, such as the natural polysaccharides or polylactic acid, polyglycolic acid, polyorthoesters, or polyanhydrides, alone or within an appropriate enteric coating, for example, as described by EPA 0 333 523 by The UAB Research Foundation and Southern Research Institute. With the exception of this synthetic polymeric system and two protein-based systems, the proteinoid microspheres described in U.S. Patent Nos. 4,925,673, 4,983,402, and 4,976,968 to Steiner and Rosen and the zein microspheres described in PCT application WO 91/06287 by Enzytech, none of these systems is stable to conditions in the stomach. Only the proteinoid system is designed particularly to be stable to the lower pH of the stomach and unstable to the higher pH of the blood.

The most desirable delivery system from a manufacturing standpoint is one that is self assembling from simple, chemically defined precursors, which are stable over the pH, temperature and solvent range of interest, yet resistant to rapid protease attack. Ideally, these precursors are economical and available, using available technology, in production volume.

WO 93/18754

PCT/US93/02245

-3-

One of the best known self-assembling encapsulation systems is the amino acid polymer self-assembling microcapsules first pioneered by Dr. Sidney Fox (Molecular Evolution and the Origin of Life). The initial experiments were with "proteinoids", a "linear thermal condensation polymer of a mixture of naturally occurring amino acids", the same system subsequently patented by Steiner and Rosen for drug delivery. While this did in fact demonstrate a capacity for self-organization under conditions plausible for early evolution, the behavior of the system is undesirable as an encapsulation technology. The stochastic nature of the precursors and the further stochastic nature of self assembly of linear polymers are inappropriate for a controllable process.

Accordingly, there remains a significant economic and medical need for an effective method for the oral delivery and release of therapeutic agents which are poorly absorbed by or unstable in the gastrointestinal tract.

It is therefore an object of the present invention to provide a system for drug delivery.

It is a further object of the present invention to provide a system which, by intentional modifications to its structure, can be made to be stable or unstable in a variety of physiological conditions.

It is another object of the present invention to provide a system which is self-assembling and can be manufactured economically from currently available reagents.

Summary of the Invention

Drug delivery systems have been developed based on the formation of diketopiperazine microparticles. In the preferred embodiment the microparticle is stable at low pH and disintegrates at a pH of about

WO 93/18754

-4-

6.5 or greater, and is particularly useful for oral drug delivery. In the most preferred embodiment the microparticles are formed in the presence of the drug to be delivered, for example, insulin or heparin. The diketopiperazine microparticles are preferably formed in the presence of the drug to be encapsulated by acidification of weakly alkaline solutions of a diketopiperazine derivative that contains one or more carboxylic acid groups.

Examples demonstrate that encapsulation and administration of insulin in rats results in subsequent control of blood glucose. Encapsulation of heparin also results in controlled release of heparin under physiological conditions and inhibits blood coagulation.

Brief Description of the Drawings

Figure 1 is a schematic of the synthesis of 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine.

Figure 2a is a graph of the average percent reduction in blood glucose levels measured in mg/dl for four and five subjects, respectively, receiving 1 ml of encapsulated insulin/kg of body weight at various time intervals (hours).

Figures 2b is a graph of the average percent reduction in blood glucose measured in mg/dl over time (hours), following administration of the microparticles not containing insulin.

Figure 3a is a graph of the clotting time (seconds) as % of baseline over time (minutes) for plasma from animals which have received encapsulated heparin by oral gavage.

Figure 3b is a graph of the clotting time (seconds) as % of baseline over time (minutes) for plasma from animals which have received microparticles not containing heparin by oral gavage.

Detailed Description of the Invention

The present invention is a drug delivery system using diketopiperazines to form microparticles encapsulating a drug to be delivered.

As used herein, the term "microparticles" includes microcapsules having an outer shell surrounding a core material; microspheres containing drug dispersed throughout the sphere; and microparticles of irregular shape. In the preferred embodiment, the microparticles are microspheres of between 0.1 to ten microns in diameter.

As used herein, "drug" and "cargo" refer to the pharmacologically active agent incorporated into the microparticles.

A clinically viable micro-encapsulation system should ideally be constructed from a chemically-defined synthetic subunit. The subunit should be of minimum complexity to permit toxicity and efficacy data to be derived to establish both safety and reproducible bioavailability with an acceptably narrow range of deviation from lot to lot (manufacturability).

The system should be self-assembling under closely controllable conditions, which conditions are benign to a range of potential drugs to be encapsulated. While the charge distribution, solubility and hydrophobicity of the cargo must clearly have some influence on assembly, a well-selected system should accept, with little modification, a range of drug cargoes. For specific cargoes, the material can optimized with minor structural manipulations.

The basic building blocks of a self-assembling system should be nonlinear in structure and should be synthesized to provide a known non-stochastic starting structure. Furthermore, the pH-dependent

WO 93/18754

PCT/US93/02245

-6-

assembly/dissassembly range should be closely controlled and defined.

There are a wide variety of sufficiently rigid structures which could constitute useful and stable self-assembling systems. Such rigid structures include amino acids and other components. The use of planar rings provides excellent stiffness, reducing the degree of freedom and therefore the variability in performance. The nucleic acids adenine and guanine are examples of the type of structures displaying significant stiffness, comprising six member rings that share an edge with five member rings. This rigidity and stability may account for their evolutionary selection as genetic code elements, imparting dimensional stability in bridging the double helix. Similarly, the use of an "end cap" on a polymer such as pyroglutamate (a pentagonal bonding agent) increases the stability of the system by aiding in "surface tiling" to provide a net curvature to a cross-linked sheet.

A preferred planar ring element that provides excellent rigidity and appropriate attachment sites for synthetic variation is dикетопиperазине or one of its substitution derivatives, including dикетоморфоліnes, dикетодіоксанes or others. A system based upon dикетопиperазине structural elements forms microparticles with desirable size distributions and pH ranges as well as good cargo tolerance. A wide range of stable, reproducible characteristics can be generated with appropriate manipulations of the attachment sites, resulting in substantial yields and excellent reproducibility.

The direct synthesis of the precursors reduces the variability in bioavailability that plagued previous systems and, perhaps more important, removes the threat of an unpredictable toxic event. Toxicity,

WO 93/18754

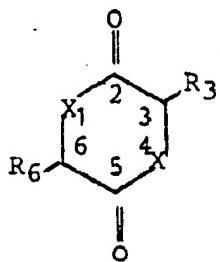
PCT/US93/02245

-7-

size, pH range and cargo capacity should be stable, experimentally verifiable parameters of the system.

Diketopiperazines.

The diketopiperazines or their substitution analogs are rigid planar hexagonal rings with opposing heteroatoms and unbonded electron pairs. One or both of the nitrogens can be replaced with oxygen to create the substitution analogs diketomorpholine and diketodioxane, respectively. Although it is possible to replace a nitrogen with a sulfur atom, this does not yield a stable structure. The general formula for diketopiperazine and its analogs is shown below.



wherein the ring atoms X at positions 1 and 4 are either O or N; and

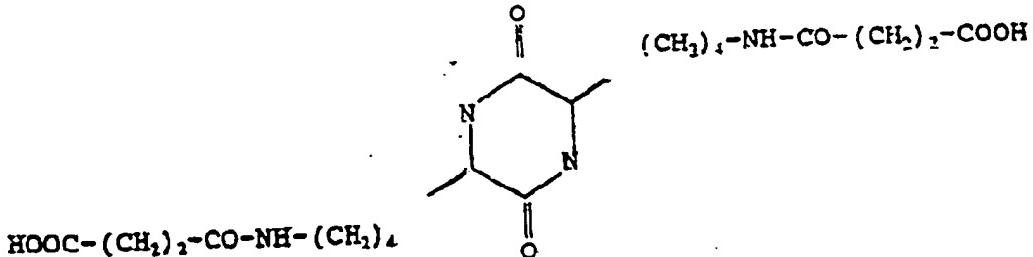
at least one of the side-chain substituents R at positions 3 and 6 contains an ionizable group such as a carboxyl group if the composition is used for oral delivery and exhibits pH-dependent assembly-disassembly, one or both must be ionizable to control release conditions. As used herein, "diketopiperazines" includes diketopiperazines and derivatives and modifications thereof falling within the scope of the above-general formula.

An example of a preferred compound, 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine, wherein X is N, and R₃ and R₄ are (CH₂)₄-NH-CO-(CH₂)₂-COOH, is shown below:

WO 93/18754

PCT/US93/02245

-3-



Methods for Synthesis of the Diketopiperazines

Diketopiperazines can be formed by cyclodimerization of amino acid ester derivatives, as described by Katchalski, et al., J. Amer. Chem. Soc. 68, 879-880 (1946), by cyclization of dipeptide ester derivatives, or by thermal dehydration of amino acid derivatives in high-boiling solvents, as described by Kopple, et al., J. Org. Chem. 33(2), 862-864 (1968), the teachings of which are incorporated herein. 2,5-diketo-3,6-di(aminobutyl)piperazine (Katchalski et al. refer to this as lysine anhydride) was conveniently prepared via cyclodimerization of N-epsilon-Z-L-lysine in molten phenol, similar to the Kopple method in J. Org. Chem., followed by removal of the blocking (Z)-groups with 4.3 M HBr in acetic acid. This route is preferred because it uses a commercially available starting material, it involves reaction conditions that are reported to preserve stereochemistry of the starting materials in the product and all steps can be easily scaled up for manufacture.

The synthesis of 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine is shown schematically in Figure 1. 2,5-diketo-3,6-di(aminobutyl)piperazine is exhaustively succinylated with succinic anhydride in mildly alkaline aqueous solution to yield a product which is readily soluble in weakly alkaline aqueous solution, but which is quite insoluble in acidic aqueous solutions. When concentrated solutions of the

WO 93/18754

PCT/US93/02245

-9-

compound in weakly alkaline media are rapidly acidified under appropriate conditions, the material separates from the solution as microparticles.

The succinylated compound, 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine, where R₃ and R₄ are (CH₂)₄-NH-CO(CH₂)₂-COOH, is shown above.

Methods for forming microparticles and encapsulating drug.

In the preferred embodiment, drug is encapsulated within microparticles by dissolving the diketopiperazine in bicarbonate or other basic solution, adding the drug in solution or suspension to be encapsulated, then solidifying the structure by adding acid, such as 1 M citric acid.

The microparticles can be stored in the dried state and reconstituted for administration to a patient. In the preferred embodiment, the reconstituted microparticles maintain their stability in an acidic medium and open up as the medium approaches physiological pH in the range of 6.5. However, materials, such as a cyclo-Lys(Z)-Lys(Z) synthetic intermediate treated with a limiting amount of HBr in acetic acid to remove one rather than both of the Z groups, are soluble in weakly acidic aqueous solutions and precipitate when the solution is made weakly alkaline with sodium bicarbonate, and could be used to form microparticles for drug delivery where it is desirable to achieve release under acidic conditions, for example, following phagocytosis and endocytosis into lysosomes. Another material that should exhibit this response to pH was obtained by heating diketopiperazine with succinic anhydride in refluxing toluene, which is expected to yield a diketopiperazine derivative N-succinylated at the 1 and 4 positions of the ring.

WO 93/18754

PCT/US93/02245

-10-

Materials that can be encapsulated.

For drug delivery, biologically active agents having therapeutic, prophylactic or diagnostic activities can be delivered. These can be organic or inorganic compounds, proteins, or a wide variety of other compounds, including nutritional agents such as vitamins, minerals, amino acids and fats. In the preferred embodiments, the materials are biologically active agents that are to be released in the circulatory system after transport from the GI tract following oral delivery. Examples include proteins and peptides (wherein protein is defined as consisting of 100 amino acid residues or more and a peptide is less than 100 amino acid residues), such as insulin and other hormones, polysaccharides, such as heparin, nucleic acids (such as antisense), lipids and lipopolysaccharides, and organic molecules having biological activity such as many of the antibiotics, anti-inflammatories, antivirals, vaso- and neuroactive agents. Specific examples include hormones, anticoagulants, immunomodulating agents, cytotoxic agents, antibiotics, antivirals, antisense, antigens, and antibodies. In some instances, the proteins may be antibodies or antigens which otherwise would have to be administered by injection to elicit an appropriate response.

In the preferred embodiment, these biological agents are unstable in gastric acid, diffuse slowly through gastrointestinal membranes, and/or are susceptible to enzymatic destruction in the gastrointestinal tract. The biological agents are encapsulated to protect them in the gastrointestinal tract prior to release in the blood stream. In the preferred embodiments, the protective material, the diketopiperazines, are not biologically active and do not alter the pharmacologic properties of the therapeutic agents.

WO 93/18754

PCT/US93/02245

-11-

The microparticles are acid stable and hence resist the acidic environment of the stomach. In addition they are resistant to enzymatic degradation in the stomach. They are believed to pass through the endothelium into the blood stream where they become soluble in the near neutral pH of the blood, liberating the pharmacologically active compound.

Examples of agents include hormones, antigens, antibiotics, steroids, decongestants, neuroactive agents, and anesthetics or sedatives. The agents can be in various forms, such as uncharged molecules or components of molecular complexes. For acidic drugs, salts of metals, amines or organic cations (e.g., quaternary ammonium) can in some cases be used. Simple derivatives of the drugs (such as ethers, esters, and amides), which have desirable retention and release characteristics, can also be used. It is not possible to have independent control of salt forms of the drug if acids and bases are being used to control the formation of the microparticles. It is not possible to have a drug molecule in the free-base or hydrochloride salt forms if the microparticles are formed by dissolving the diketopiperazine in sodium bicarbonate solutions and adding concentrated citric acid.

Imaging agents including metals, radioactive isotopes, radiopaque agents, and radiolucent agents, can also be incorporated. Radioisotopes and radiopaque agents include gallium, technetium, indium, strontium, iodine, barium, and phosphorus.

Pharmaceutical Compositions.

The microparticles can be administered in suspension or encapsulated in another material such as an enteric coating or stabilizing agent such as albumin or lactose. These materials and methods for use thereof are well known to those in the pharmaceutical industry. The pharmaceutical

WO 93/18754

PCT/US93/02245

-12-

composition may consist only of the microparticles or may further include the encapsulated compound, or other compounds. For example, it may be desirable to administer a compound that is stable to passage through the stomach that is then rapidly absorbed in one dosage in the intestine, followed by the more controlled, delayed release of the same or a different compound from the microparticles, i.e., enterically protected basic stable, neutral, soluble microcapsules, if the compound can tolerate encapsulation.

The microparticles can be administered topically, locally or systemically by parenteral administration or enteral administration.

Enteral Administration.

Microparticles having biologically active agents are preferably administered orally. These microparticles, depending on the chemical nature and size, will either be absorbed to, or passed through, the epithelial lining of the gastrointestinal tract into the bloodstream or lymphatic system.

Parenteral Administration.

Microparticles of less than five microns readily pass through a needle for intravenous administration. Suitable pharmaceutical carriers, for example, a phosphate buffered saline, are known and commercially available. Intravenous administration may be preferred for targeted delivery of incorporated compounds to phagocytic cells, for example, of antiparasitic or anti-HIV drugs, where the pathogenic agent is also selective for these cell types. Microcapsules should be stable at neutral pH and dissolve at low pH, the reverse of the oral system.

Subcutaneous, Intramuscular and Intraperitoneal Administration.

Microparticles produced as described above are small enough to be injected through a standard gauge

WO 93/18754

PCT/US93/02245

-13-

needle under the skin or into the peritoneum for subsequent release of incorporated drug. Adhesion of the microparticles to the peritoneum aids in localizing release of the incorporated drug. Microparticles can also be implanted or injected intramuscularly for immunization or other purposes where slower release into the bloodstream is desirable. Carriers such as phosphate buffer saline, or an adjuvant such as an oil, can be used as a carrier for the microparticles. Pharmaceutically acceptable carriers are known to those skilled in the art.

Topical Administration.

Microparticles are suspended in a suitable pharmaceutical carrier for administration using methods appropriate for the carrier and site of administration. For example, microparticles are administered to the eye in a buffered saline solution, approximately pH 7.4, or in an ointment such as mineral oil. The dosage will be dependent on the compound to be released as well as the rate of release. The microparticles, or aggregations of microparticles into films, disks, or tablets, with incorporated compound can be administered to the skin in an ointment or cream. Suitable pharmaceutical carriers are known to those skilled in the art and commercially available.

Sustained delivery of antibiotics or growth factors (amino acids, peptides, or protein growth factors) to open wounds is of particular therapeutic importance in a variety of medical and surgical situations including, but not limited to, thermal burns, chemical burns, surgical wounds, diabetic ulcers and vascular insufficiency.

Diagnostic Applications.

The microparticles containing radiopaque compounds, radioisotopes, or radiolucent compounds are

WO 93/18754

PCT/US93/02245

-14-

particularly suited for use in diagnostic procedures. The microparticles can be administered parenterally or enterally. Microparticles that bind to mucosal membranes are particularly preferred for these applications, especially for imaging of the nasal and pharyngeal, gastrointestinal, and genito-urinary tracts. Intravenous administration of microparticles containing imaging agents are particularly useful for imaging liver, spleen or lung.

Targeted Administration.

Delivery to specific cells, especially phagocytic cells and organs.

Phagocytic cells within the Peyer's patches appear to selectively take up microparticles administered orally. Phagocytic cells of the reticuloendothelial system also take up microparticles when administered intravenously. Microparticles of less than five microns diameter can be injected without embolic complications. Endocytosis of the microparticles by macrophages can be used to target the microparticles to the spleen, bone marrow, liver and lymph nodes.

The charge or lipophilicity of the microparticle is used to change the properties of the protein carrier. For example, the lipophilicity of the inner surface of the microcapsules can be modified by linking lipophilic groups to increase solubility of some drugs, thereby increasing drug cargo capacity. Other modifications can be made before or after formation of the microparticle, as long as the modification after formation does not have a detrimental effect on the incorporated compound.

Administration of the Microparticles to a Patient.

In the preferred embodiment, the microparticles are stored lyophilized or encapsulated in standard gel capsule materials, for subsequent oral administration.

WO 93/18754

PCT/US93/02245

-15-

The dosage is determined by the amount of encapsulated drug, the rate of release within the gastrointestinal tract, and the pharmokinetics of the compound.

In some embodiments, the microparticles can also be administered by injection, either intravenous, intramuscular, or subcutaneous, topically, or by means of a transdermal patch where release is activated by contact with the low pH of the skin (reverse stability formulation).

The present invention will be further understood by reference to the following non-limiting examples of the preparation and administration of diketopiperazine microparticles containing insulin.

Example 1: Preparation of diketopiperazine microparticles.

cyc^{YQ}-Lys(Z)-Lys(Z)

(Cyclodimerization of N-epsilon-(Z)-L-lysine)

The method of synthesis is shown schematically in Figure 1.

The methods of Ephraim Katchalski, Issac Grossfeld, and Max Frankel, "Synthesis of lysine anhydride" Journal of the American Chemical Society 68, 879-880 (1946) and Kenneth D. Kopple and Hayop G. Ghazarian, "A convenient synthesis of 2,5-diketopiperazines" Journal of Organic Chemistry 33, 862-864 (1968) were adapted to use as follows. Katchalski et al describe the synthesis of the target compound by a different synthetic route; Kopple, et al describe a synthetic method similar to that used herein, but not using a lysine-based dipeptide nor yielding the same target compound. The letter "Z" is used to designate the benzyloxycarbonyl or carbobenzoxy group used to protect the amino group.

N-epsilon-Z-L-lysine

N-epsilon-Z-L-lysine (Sigma Chemical Co, St. Louis, MO, 50 grams) was cyclized as follows. The compound was placed with 250 grams of crystalline

-16-

phenol in a 500 mL resin reaction kettle under a gentle flow of nitrogen gas (prepurified grade). The temperature of the reaction mixture was raised to 175°C (heating mantle) and held at that temperature under nitrogen for 18 hours. The reaction kettle was removed from the heating mantle and allowed to cool until the outside of the vessel was not warm to the touch and crystals were just beginning to form in the reaction mixture. The reaction mixture was then mixed with 1.5 L anhydrous ether with stirring to precipitate a fine, white powder. This precipitate was collected on a sintered glass funnel (coarse grit) and washed on the filter with anhydrous ether. After air drying on the filter, the product (JG47) weighed 33.7 grams. A portion of the product (5 grams) was separated for analysis.

cyclo-Lys(Z)-lys(z)

The material was dissolved in 50 mL of hot glacial acetic acid and the solution was filtered to remove a small amount of insoluble material. On cooling, a solid crystallized from the acetic acid solution. This material was collected by filtration, then suspended in 200 mL 1:1 water:methanol. The suspension was brought to gentle reflux, then allowed to stand at room temperature for 2 days. The purified product (JG48) was collected by filtration and air dried on the filter. This procedure yielded 3.7 grams of purified cyclo-Lys(Z)-lys(z).

2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine Dihydrobromide

(Deprotection of cyclo-Lys(Z)Lys(Z))

To deprotect and leave terminal amino groups on the side chains, twenty grams of cyclo-Lys(Z)-Lys(z) (JG47, finely powdered) was suspended in 50 mL of glacial acetic acid, with stirring. To this suspension was added 50 mL of 4.3 M HBr in glacial acetic acid to remove the Z-group. For a time there

-17-

was a rapid evolution of gas (carbon dioxide) and the solid dissolved almost completely; then a solid product began to separate from the reaction mixture.

Two hours after addition of the HBr solution, 150 mL of anhydrous ether was added to the mixture to complete precipitation of the product. The precipitated product was washed repeatedly with ether, then dried under a stream of dry nitrogen. The crystalline residue was used directly for succinylation.

2,5-diketo-3,6-di(4-aminobutyl)piperazine

The cyclo-Lys-Lys dihydrobromide from the preceding procedure was acylated into 2,5-diketo-3,6-di(4-aminobutyl)piperazine with succinic anhydride. First, it was dissolved in 200 mL of solution made by saturating water with sodium bicarbonate at room temperature. This dissolution was done slowly so that carbon dioxide gas could escape without causing the mixture to foam out of the reaction vessel. The solution was filtered to remove a small amount of insoluble material and the filter was washed with an additional 50 mL of saturated sodium bicarbonate solution which was added to the filtrate.

The solution was stirred with an efficient magnetic stirrer and with continuous monitoring of the pH using a glass electrode. The initial pH was 8.7. Succinic anhydride (30 grams) was added in ten portions. Each time the pH of the reaction mixture fell to 7.5, it was readjusted to 8.7 with 4 M NaOH. The pattern of adding succinic anhydride and readjusting the pH was continued until all of the succinic anhydride was dissolved and the final pH stabilized (about half an hour after addition of the last portion of succinic anhydride).

To precipitate the microparticles, citric acid (10 grams) was added to the reaction mixture, then the pH was slowly adjusted to 2.2 with concentrated HCl.

-18-

(There is a vigorous evolution of carbon dioxide during this process, which is controlled by slow addition of the HCl). At about pH 3-3.5, a solid product began to separate from the solution. At pH 2.2 the solution was filled with fine particles. The mixture was placed in the refrigerator overnight, then the product was collected by filtration, washed with water, and air dried on the filter. The yield was 11.7 grams of off-white powder (JG52).

A small sample of the product was dissolved in the minimum volume of water at the boiling point. The solid that separated on cooling was collected by centrifugation, washed with water, then lyophilized from a centrifugal pellet (JG77).

Example 2: Suppression of blood glucose by oral administration of insulin.

Method:

Porcine insulin (Sigma Chemical Co., St. Louis, MO, specific activity of approximately 26 U/mg) was encapsulated in 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine by dissolving the piperazine in a saturated sodium bicarbonate solution to form a 125 mg piperazine/mL solution, then mixing this solution with an equal volume of a 1 M citric acid solution containing the insulin to be incorporated in a concentration of 20 mg insulin/ml. This yields a suspension of approximately 67.5 mg microparticles/ml.

In total, nine (9) male rats, each weighing approximately 250 g and having a normal blood glucose level, received by oral gavage encapsulated insulin administered at a calculated dosage of between 1.25 and 2.0 mg insulin/kg of body weight. For controls, rats received an amorphous precipitate of the polymer and insulin prepared so that no insulin was encapsulated in spheres, but the concentration of insulin in the final solution was the same as that

WO 93/18754

PCT/US93/02245

-19-

used in the original preparation. When this suspension was administered by oral gavage to four control rats, at a dose of 1 ml of suspension/kg of body weight, no significant decrease in blood glucose was noted. Additionally, subjects were administered insulin subcutaneously as both an aqueous solution and in the form of an amorphous precipitate to demonstrate the biological activity of the insulin itself.

Blood glucose levels were measured on samples taken from the tail at various times after treatment and measured as mg of glucose/dl of blood using one drop of tail blood squeezed onto a Glucofilm strip.

Results: Figure 2a presents the average percent reduction in blood glucose levels measured in (mg/dl) for nine subjects receiving 1 ml of encapsulated insulin suspension at a concentration of 10 mg/kg of body weight at various time intervals. The encapsulated insulin produced a marked fall in blood glucose levels when administered orally. Oral administration of an amorphous precipitate solution of the polymer with the same amount of insulin failed to produce a significant change in blood glucose levels, as shown by Figure 2b. The same solution injected subcutaneously produced a characteristic drop in blood glucose; as did an injection of pure insulin in an aqueous solution. The absence of a pharmacologic effect with oral administration of unencapsulated insulin is consistent with the findings of other studies in the literature done at higher doses in both animals and humans. Figure 2a demonstrates that blood glucose levels are returning to pre-administration values at 240 minutes.

Example 3: Inhibition of clotting in blood by microencapsulated heparin.

Heparin (Sigma Chemical Co., St. Louis, MO, specific activity approximately 26 U/mg) was encapsulated as described above by dissolving the

WO 93/18754

PCT/US93/02245

-20-

disuccinyl derivative of 2,5-deketo-3,6-di(4-aminobutyl)piperazine in a saturated sodium bicarbonate solution to a concentration of 120 mg piperazine/mL of solution, then mixing this with an equal volume of 1 M citric acid containing 100 mg sodium heparin/mL citric acid.

The final suspension contained 50 mg of heparin/ml of suspension. Of this, approximately 20% was encapsulated, yielding a theoretical maximum concentration of encapsulated heparin of 10 mg of heparin per ml of suspension.

The solution containing encapsulated heparin was administrated to eight rats weighing approximately 250 grams, by oral gavage. The rats were fasted overnight prior to treatment. Each rat received 1 ml of suspension per kg of body weight. Additionally, a suspension of microcapsules formed in 1 M citric acid with no heparin present was administered to a group of four (4) control rats.

At zero minutes, 60 minutes, 120 minutes, 240 minutes, and 360 minutes, blood was drawn into a citrated syringe in a ratio of 9:1. The blood was immediately centrifuged and the plasma assayed using the APTT assay with standard reagents. The results are shown in Figure 3a. The results clearly indicate that the oral administration of heparin was effective in prolonging clotting times.

Figure 3b shows the results of the control group. The results clearly indicate that the microparticles themselves do not appreciably effect clotting time.

Modifications and variations of the method of the present invention will be obvious to those skilled in the art from the foregoing detailed description. Such modifications and variations are intended to come within the scope of the appended claims.

WO 93/18754

PCT/US93/02245

-21-

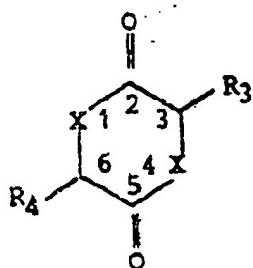
We claim:

1. A microparticulate system for drug delivery comprising:

diketopiperazine microparticles incorporating a biologically active agent selected from the group consisting of proteins, peptides, polysaccharides, lipids, lipopolysaccharides, nucleic acids and other biologically active organic molecules,

wherein the microparticles are stable at a first defined pH due to association and precipitation of the diketopiperazines and unstable at a second defined pH due to dissociation of the diketopiperazines.

2. The system of claim 1 wherein the diketopiperazine has the general structure



wherein the ring atoms X at positions 1 and 4 are either O or N; and

at least one of the side-chain substituents R at positions 3 and 6 contains a carboxyl group

3. The system of claim 2 wherein the structure is formed from amino acids selected from the group consisting of glutamic acid, aspartic acid, lysine, ornithine and diaminopropionic acid.

4. The system of claim 3 wherein the structure is selected from the group consisting of 2,5-diketo-3,6-di(aminobutyl)piperazine and 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine.

WO 93/18754

PCT/US93/02245

-22-

5. The system of claim 1 wherein the microparticles are stable at acidic pH and unstable at a more basic pH.

6. The system of claim 1 wherein the microparticles are unstable at acidic pH and stable at a more basic pH.

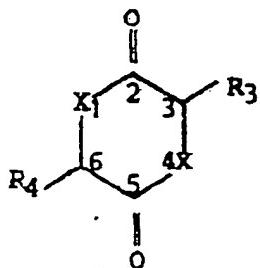
7. The system of claim 1 wherein the biological agent is insulin.

8. The system of claim 1 wherein the biological agent is heparin.

9. A method for making a microparticulate system for drug delivery comprising forming diketopiperazines in a solution with a first defined pH at which the diketopiperazines are soluble,

adding to the diketopiperazine solution a biologically active agent selected from the group consisting of proteins, peptides, polysaccharides, lipids, lipopolysaccharides, nucleic acids and other biologically active organic molecules, imaging agents, and cell specific targeting agents, in a solution having a second defined pH precipitating the diketopiperazines to form microparticles of the diketopiperazines containing the biologically active agent.

10. The method of claim 9 wherein the diketopiperazine has the general structure



WO 93/18754

PCT/US93/02245

-23-

wherein the ring atoms X at positions 1 and 4 are either O or N; and at least one of the side-chain substituents R at positions 3 and 6 contains a carboxyl group.

11. The method of claim 9 wherein the structure is formed from amino acids selected from the group consisting of glutamic acid, aspartic acid, lysine, ornithine and diaminopropionic acid cyclized by blocking free β -amino groups, heating in hot phenol under nitrogen at a temperature equivalent to 175°C, and removing the blocking groups.

12. The method of claim 11 further comprising succinylating a side chain of the diketopiperazine having a free amino group.

13. The method of claim 9 wherein the structure is selected from the group consisting of 2,5-diketo-3,6-di(aminobutyl)piperazine and 2,5-diketo-3,6-di(4-succinylaminobutyl)piperazine.

14. A method for administering a biologically active agent to a patient comprising providing the agent selected from the group consisting of proteins, peptides, polysaccharides, lipids, lipopolysaccharides, nucleic acids and other biologically active organic molecules, imaging agents, and cell specific targeting agents, in combination with microparticles formed of diketopiperazines, wherein the microparticles are stable at a first defined pH due to association and precipitation of the diketopiperazines and unstable at a second defined pH due to dissociation of the diketopiperazines.

15. The method of claim 14 wherein the microparticles further comprise compounds selected from the group consisting of stabilizers of the biologically active agents and non-encapsulated biologically active compounds.

WO 93/18754

PCT/US93/02245

-24-

16. The method of claim 14 wherein the agent is selected from the group consisting of hormones, anticoagulants, immunomodulating agents, cytotoxic agents, antibiotics, antivirals, antisense, antigens, and antibodies.

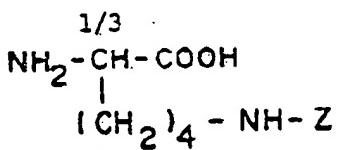
17. The method of claim 14 wherein the microparticles are further encapsulated within an enteric coating.

18. The method of claim 14 wherein the microparticles are stable at acidic pH and unstable at neutral or basic pH.

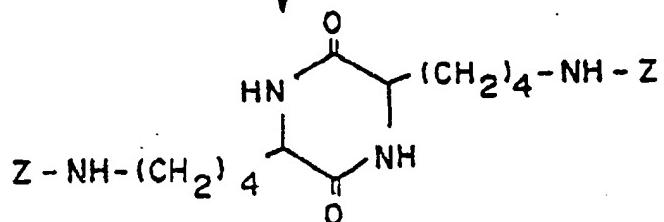
19. The method of claim 14 wherein the microparticles are unstable at acidic pH and stable at neutral or basic pH.

20. The method of claim 14 wherein the biological agent is insulin.

21. The method of claim 14 wherein the biological agent is heparin.

N-ε-Z-L-lysine

↓ MOLTEN PHENOL, 175° C

cyclo-o-Lys(Z)-Lys(Z)

↓ HBr in glacial acetic acid

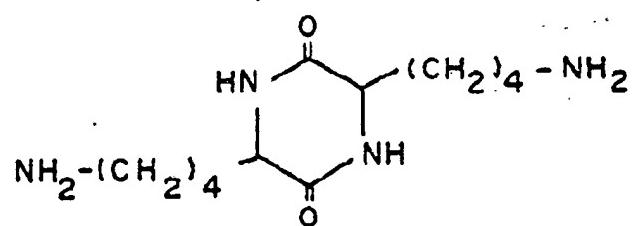
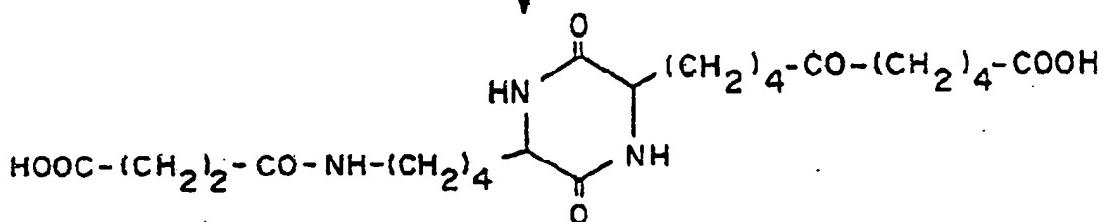
2, 5-Diketo-3, 6-di(4-aminobutyl) piperazine↓ SUCCINIC ANHYDRIDE,
WEAKLY ALKALINE AQUEOUS
MEDIA2, 5-Diketo-3, 6-di(4-succinylaminobutyl) piperazine

FIG. 1

WO 93/18754

PCT/US93/02245

2/3

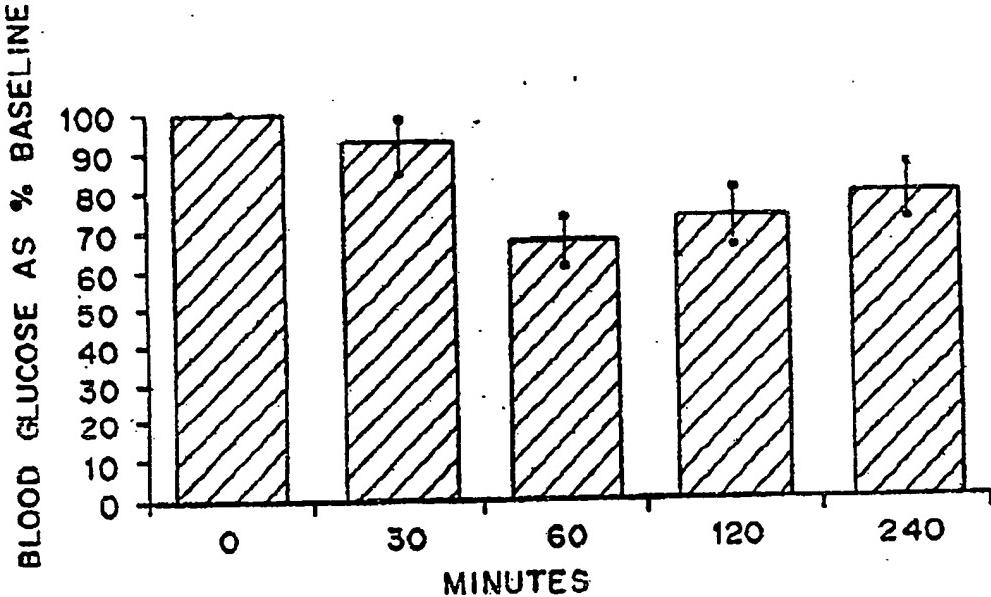


FIG. 2a

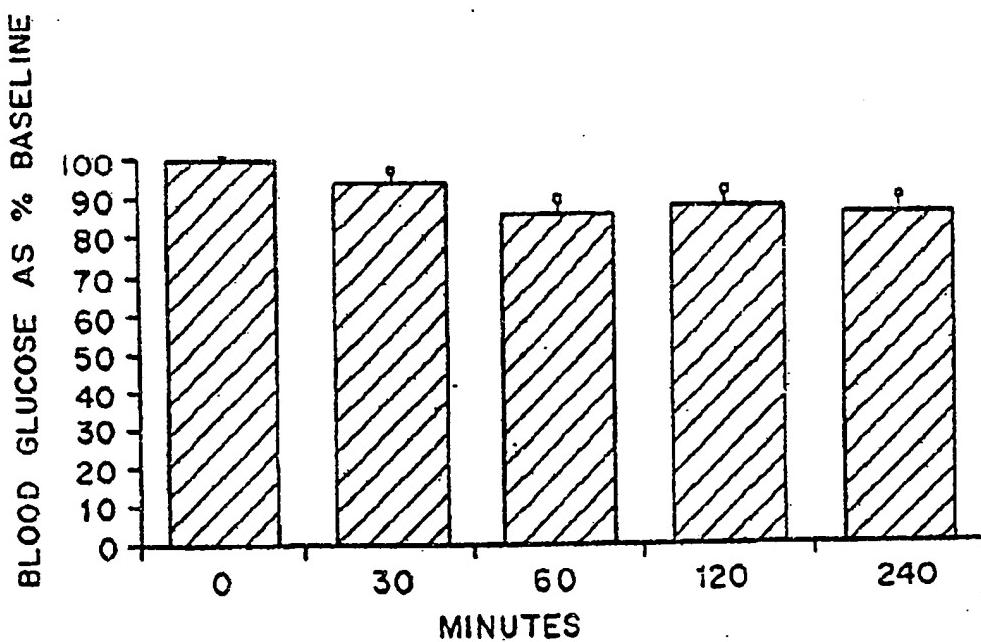


FIG. 2b

WO 93/18754

PCT/US93/02245

3/3

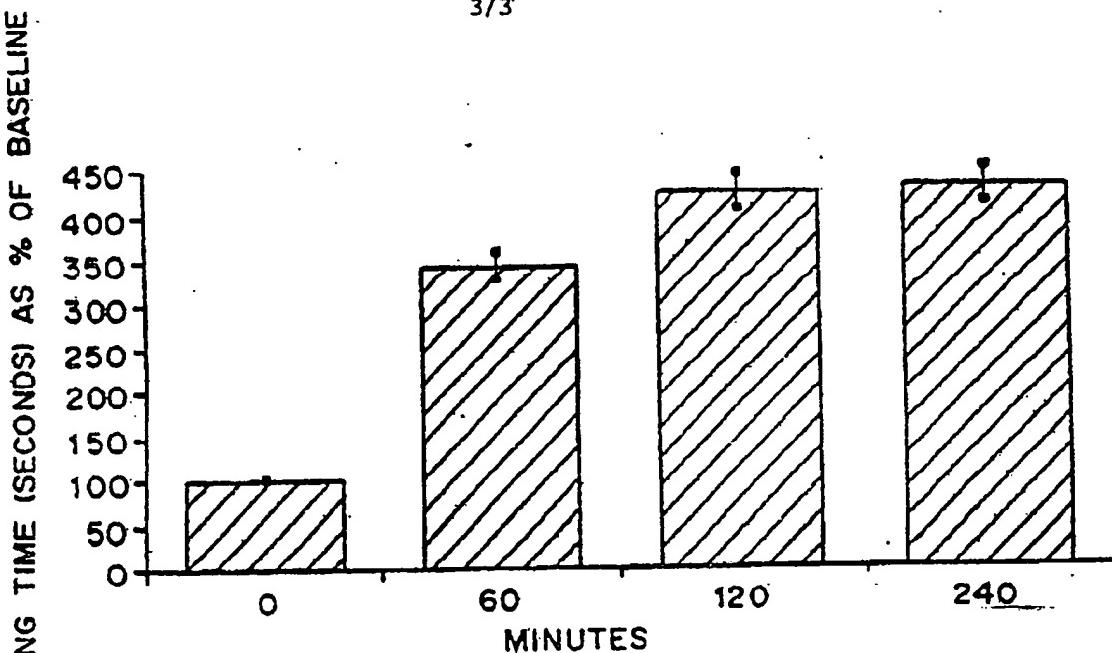


FIG. 3a

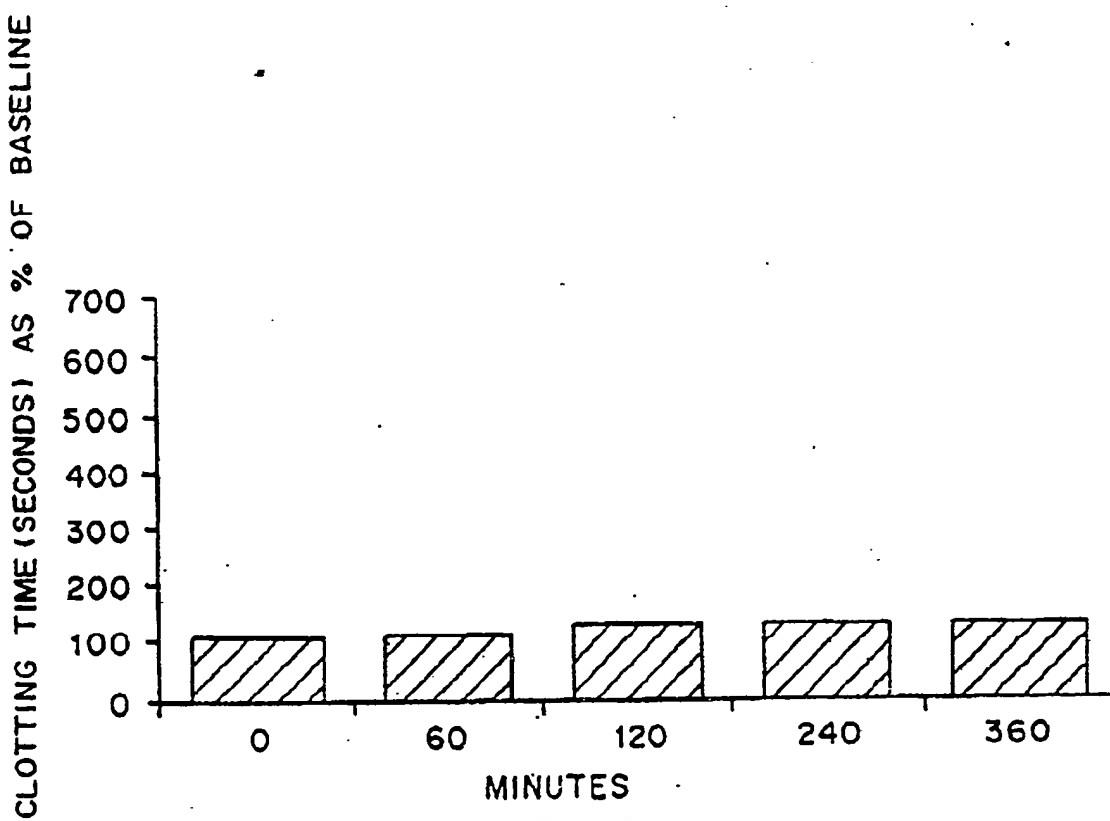


FIG. 3b

INTERNATIONAL SEARCH REPORT

PCT/US 93/02245

International Application No

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all)⁴

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K9/16

II. FIELDS SEARCHED

Minimum Documentation Searched⁵

| Classification System | Classification Symbols |
|-----------------------|------------------------|
| Int.Cl. 5 | A61K |

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched⁶III. DOCUMENTS CONSIDERED TO BE RELEVANT⁷

| Category ⁸ | Citation of Document, If with indication, where appropriate, of the relevant passages ¹² | Relevant to Claim No. ¹³ |
|-----------------------|---|-------------------------------------|
| Y | EP, A, 0 350 246 (TAKEDA CHEMICAL INDUSTRIES, LTD.) 10 January 1990 see the whole document see page 9; example 7 --- | 1-21 |
| Y | JOURN. AM. CHEM. SOC. vol. 68, May 1946, GASTON (US) pages 879 - 880 E. KATCHALSKI ET AL. 'synthesis of lysine anhydride' cited in the application see the whole document --- | 1-21 -/- |

¹⁰ Special categories of cited documents:

- ¹¹ "A" document defining the general state of the art which is not considered to be of particular relevance
- ¹² "E" earlier document but published on or after the international filing date
- ¹³ "I" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reasons (as specified)
- ¹⁴ "O" document referring to an oral disclosure, use, exhibition or other means
- ¹⁵ "P" document published prior to the international filing date but later than the priority date claimed

¹⁶ "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention¹⁷ "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step¹⁸ "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art¹⁹ "Z" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

24 JUNE 1993

Date of Mailing of this International Search Report

17.93

International Searching Authority

EUROPEAN PATENT OFFICE

Signature of Authorized Officer

BENZ K.F.

PCT/US 93/02245

International Application No

| III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET) | | |
|--|---|------------------------|
| Category* | Citation of Document, with indication, where appropriate, of the relevant passages | Relevant to Claims No. |
| Y | JOURN. ORG. CHEM. vol. 33, no. 2, February 1968, EASTON (US) pages 862 - 864 K.D. KOPPLE ET AL. 'a convenient synthesis of 2,5-piperazinediones' cited in the application see the whole document --- | 1-21 |
| A | FR,A,2 145 555 (BEECHAM GROUP LIMITED) 23 February 1973 see the whole document --- | 9 |
| A | GB,A,1 155 036 (BROWN) 11 June 1969 see page 2, line 64 - line 106 see page 4, line 41 - line 78 see claims 1,5 --- | 1-21 |
| A | EP,A,0 284 039 (CHUGAI SEIYAKU K K) 28 September 1988 --- | 1-21 |
| A | GB,A,1 255 805 (CHEMISCHE WERKE ALBERT) 1 December 1971 see page 1, line 50 - line 69 see page 4; example 1 --- | 1-21 |
| A | US,A,4 976 968 (STEINER) 11 December 1990 cited in the application ----- | 1-21 |

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US93/02245

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.:

because they relate to subject matter not required to be searched by this Authority, namely:

Remark: Although claims 14-21 are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the composition.

2. Claims Nos.:

because they relate to parts of the International application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. Claims Nos.:

because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

 The additional search fees were accompanied by the applicant's protest. No protest accompanied the payment of additional search fees.

**ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.**

US 9302245
SA 71536

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EPO file on The European Patent Office is in no way liable for those particulars which are merely given for the purpose of information. 24/06/93

| Patent document cited in search report | Publication date | Patent family member(s) | | Publication date |
|--|------------------|---|--|--|
| EP-A-0350245 | 10-01-90 | JP-A- | 2124814 | 14-05-90 |
| FR-A-2145555 | 23-02-73 | GB-A- AU-B- AU-A- BE-A- CA-A- DE-A, B, C NL-A- SE-B- US-A- | 1377074 462878 4450572 786026 973093 2234474 7209574 385770 3792159 | 11-12-74 10-07-75 17-01-74 08-01-73 19-08-75 25-01-73 16-01-73 26-07-76 12-02-74 |
| GB-A-1155036 | 11-06-69 | None | | |
| EP-A-0284039 | 28-09-88 | AU-B- AU-A- DE-A- JP-A- US-A- | 603878 1351788 3872739 64000030 5057317 | 29-11-90 29-09-88 20-08-92 05-01-89 15-10-91 |
| GB-A-1255805 | 01-12-71 | DE-A- DE-A- AT-A- AU-A- BE-A- FR-A- FR-A, B GB-A- NL-A- SE-B- US-A- US-A- AT-A- FR-M- US-A- | 1810705 1617418 300190 5220073 725125 1605467 2181717 1417323 6818068 384797 4576947 4515795 301759 8339 3864469 | 13-08-70 25-03-71 15-06-72 19-09-74 09-06-69 14-05-76 07-12-73 10-12-75 18-06-69 24-05-76 18-03-86 07-05-85 15-08-72 14-12-70 04-02-75 |
| US-A-4976968 | 11-12-90 | None | | |